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Molecular Analysis of White Clover Population Structure in Grazed Swards during Two Growing Seasons

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ABSTRACT

White clover (*Trifolium repens* L.) populations persist for years in grazing lands primarily through clonal growth, yet retain high genetic variability. This study was conducted to determine how clone structure dynamics affected intraspecific genetic variation of white clover at three pasture sites. Up to 37 trifoliate leaf samples were taken monthly by resampling specific points in four 1.2 x 1.2 m area quadrats from April to September for 2 yr; random amplified polymorphic DNA (RAPD) profiles of 1160 and 973 samples, in 1997 and 1998, respectively, were analyzed. Significantly more clones were sampled in 1997 (162) than in 1998 (58) ($P < 0.0001$). The majority of clones were not detected more than once during each year. The soil water content was significantly lower in 1998 than in 1997 ($P < 0.0001$). The number of sampled clonal members in quadrats ranged from 0.5 to 12.8 across both years on the three pastures. Within-population analysis of molecular variances (AMOVA) by date for the three pastures ranged from 15 to 74% and 46 to 80% in 1997 and 1998, respectively, indicating low to medium genetic diversity in the populations. The fraction of clonal samples relative to the total number of samples ranged from 0.03 to 0.78 in 1997 and 0.04 to 0.33 in 1998. Higher numbers of clonal members appeared to reduce genetic diversity; however, this was offset by rapid turnover of clones. We conclude that genetic variability of white clover is dynamic at the local scale, which contributes to its long-term persistence in grazing lands.

WHITE CLOVER is an important functional component of temperate grazed ecosystems because of symbiotic nitrogen fixation and its high nutritional quality (Caradus et al., 1996) as an animal feed. White clover is a stoloniferous, obligately outcrossing, tetraploid species. It flowers prolifically during the growing season, and even under grazing produces significant levels of viable seed that end up in the soil (Chapman and Anderson, 1987; Charlton, 1977).

In spite of the presence of viable white clover seed in the soil (Tracy and Sanderson, 2000), few of these seeds germinate and even fewer seedlings become established in grasslands (Barratt and Silander, 1992; Brink et al., 1999; Chapman and Anderson, 1987; Fothergill et al., 1997; Grime et al., 1988). However, naturalized white clover populations persist for many decades in grazed swards at northern midlatitudes. White clover persistence in pastures where commercial seed is not applied is due to two primary mechanisms: (i) New plants (genotypes made up of one or more rooted stolons and trifoliates) can be established through rare germination and subsequent rare seedling recruitment in the spring (Chapman, 1983; Fothergill et al., 1997; Grime et al., 1988); and (ii) plants can increase in size by clonal propagation via stolon nodes (Chapman, 1983). When nodes within a stolon die, resulting fragments become separate plants of the same genotype. Because white clover spreads primarily by vegetative propagation and because plants are thought to continuously propagate, one might expect many clonal patches and therefore low genetic variability within pastures. Although a white clover genotype could potentially dominate a grassland by fragmenting into many clonal plants (Cahn and Harper, 1976), the largest diameter clone reported was less than 6 m (Harberd, 1963).

Clonal patches generally range in size from <1 to 5

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Abbreviations: AMOVA, analysis of molecular variance; HU, Huntingdon County; JU, Juniata County; MI, Mifflin County; RAPD, random amplified polymorphic DNA; Φ_{st}, correlation of random genotypes within populations relative to that of random pairs of genotypes drawn from the whole species.

m^2, and patch size is limited by site-specific conditions (Cahn and Harper, 1976; Harberd, 1963). Genetic variability of white clover can be high at the local (Burdon, 1980) and regional (Gustine and Huff, 1999) scales. It is unclear how white clover maintains high intraspecific genetic diversity in the face of partial dominance by a single successful genotype. Vague explanations such as "undefined selective or biotic forces" frequently are cited along with infrequent seed germination and seedling recruitment (Burdon, 1980; Cahn and Harper, 1976).

The purpose of our study was to investigate how clone structure dynamics of white clover populations affected intraspecific genetic variation during two growing seasons in three rotationally stocked swards in the northeastern USA. Random amplified polymorphic DNA profiles have been used to characterize genetic variability, clonal structure, and population structure in several plant species (Buso et al., 1998; Gustine and Huff, 1999; Huff et al., 1998; Palacios and Gonzales-Candelas, 1997; Sydes and Peakall, 1998). Random amplified polymorphic DNA profile determination is based on unknown DNA sequences that are stable to the environment under field conditions and are inherited in a dominant Mendelian fashion. These markers do not distinguish between heterozygous and homozygous alleles, as do isozyme markers, but a much greater number of markers can be generated. With this approach, we could identify genotypes of sampled plants, assign samples to a clone, record the temporal occurrence of clones, follow temporal changes in genetic variance, and estimate genetic diversity in white clover populations.

MATERIALS AND METHODS

The white clover populations used in this study were part of three managed permanent pastures in the ridge and valley physiographic region of Pennsylvania (Table 1). The sites were similar in elevation but varied in soil types (Table 1). The rotationally stocked swards were not treated with chemical fertilizers. The pastures had been grazed for at least 5 yr since the previous seeding, and the white clover populations studied presumably had developed from the viable seed pool (as defined by Silvertown and Lovett Doust, 1993) of naturalized clover. The pastures in Juniata (JU) and Mifflin (MI) Counties consisted of 10 to 60% white clover and 10 to 80% grasses, which were composed of tall fescue (*Festuca arundinacea* Schreb.), quackgrass [*Elytrigia repens* (L.) Desv. ex Nevski], and Kentucky bluegrass (*Poa pratensis* L.). The pasture in Huntingdon County (HU) consisted of 70% reed canarygrass (*Phalaris arundinacea* L.) and less than 25% white clover. The distribution of white clover genotypes was studied in four permanent 2.8-m^2 quadrats. Quadrats 3 and 4 were in a flat, well-drained paddock about 2 m above and 100 m away from quadrats 1 and 2. Each of the pair of locations was separated by 10 to 30 m. The locations were laid out in the spring before growth of white clover plants was initiated.

The mean floristic composition and bare ground areas (sward gap area) were determined from estimates made in 10 subquadrats that had not been sampled for white clover. The extent of vegetative cover (composed of white clover, grasses, and other species) and the sward gap area was estimated visually at each sampling date. The weed cover in the quadrats on the three pastures ranged from 0 to 50% of the sward.

Soil water data were recorded for each sampling date with a Trase[1] time domain reflectometer (Soilmoisture Equipment Corp., Santa Barbara, CA) using 15 cm waveguides. The mean volumetric soil water was determined from readings taken at eight points near the perimeter of the quadrat and at two unsampled subquadrats within the quadrat. The field capacities for Hagerstown, Edom, and Clarksburg soils were 0.16 to 0.24, 0.12 to 0.16, and 0.16 to 0.2 $m^3\ m^{-3}$ of water in the surface 15 cm, respectively (USDA, 1981). The total precipitation from January through August, 1997, was 606 mm at the HU site, and 646 mm (25 mm above normal) at the JU and MI sites. For the same 8 mo in 1998, total precipitation was 802 mm at the HU site and 962 mm (290 mm above normal) at the JU and MI sites (data not available for September through December, 1998). Normal precipitation for January through August for the HU site was not available.

The four quadrats on each of the pastures were sampled on five dates from May to September at the points indicated on the sampling grid shown in Fig. 1. The grid was in place only during sampling. We collected 2133 leaf samples, or 48% of the maximum of 4440 samples that could have been collected during the study (had there been a stolon with a trifoliate leaf at each sampling point for every quadrat in both years). The total numbers of samples collected in 1997 and 1998 were 1160 and 973, respectively. Trifoliate leaf samples were taken from up to 37 sampling points in a quadrat at each date. Each sample for genomic DNA analysis consisted of one to four trifoliate leaves from the same stolon. If no stolon was present within 2 cm of the sampling point, a sample could not be taken; if more than one stolon was present at the sampling point, trifoliate leaves were collected from a randomly selected stolon. Since we did not mark stolons, we did not know if a plant was sampled on more than one date. If a stolon had no trifoliate leaves, the plant was not sampled. Leaf samples were stored on ice at the time of collection,

[1] Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Table 1. Site characteristics at three Pennsylvania pastures where white clover populations were sampled.

Location	Lat. N	Long. W	Elevation	Annual Precip.	Annual mean temp.	Soil Series	Taxonomic Name	Seeding Year and Species	Year Pasture Established
			m	mm	°C				
HU†	40° 35′	78° 08′	305	1031	9.7	Hagerstown	Fine, mixed, semiactive, mesic Typic Hapludalfs	Fall 1992, Ladino wc,‡ Reed canarygrass	1985
						Clarksburg	Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs		
JU	40° 34′	77° 16′	189	1095	10.3	Edom	Fine, illitic, mesic Typic Hapludalfs	Never seeded in wc	1990
MI	40° 38′	77° 38′	232	1095	10.3	Hagerstown	Fine, mixed, semiactive, mesic Typic Hapludalfs	Never seeded in wc	1991

† HU, Huntingdon County; JU, Juniata County; MI, Mifflin County.
‡ wc, white clover.

processed, and RAPD profiles generated (Gustine and Huff, 1999). Because of the time required to perform RAPD analyses, only a single DNA extraction was done for each sample; however, repeated polymerase chain reaction amplifications of genomic DNA samples consistently produced the same markers. Polymerase chain reactions of genomic DNA, gel electrophoresis, and ethidium bromide staining was performed, according to Gustine and Huff (1999). Gels were documented with the Kodak DC120 digital camera and bands detected with Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY). Some genomic DNA preparations that did not yield usable RAPD profiles were precipitated in 2 *M* NaCl to remove polysaccharides (Sambrook et al., 1989). Genomic DNA from this purification step gave useful RAPD profiles. Polymerase chain reactions were performed at least twice on each sample, and only repeatable bands were scored.

Three primers (OPA08, OPB14, and OPH12; Operon Technologies, Alameda, CA) used by Gustine and Huff (1999) were used for this study. The initial RAPD profile data set consisted of 32 molecular markers found in the 2133 samples. From these, 28 markers were selected that were represented on all of the dates. Marker size was determined by comparison with the 100-bp ladder from Life Technologies (Gaithersburg, MD) and ranged from 0.275 to 1.45 kilobase pairs (kb) in length. Markers were scored as present (1) or absent (0). No monomorphic markers were present in the data set. The number of DNA markers defining a clone ranged from 3 to 18 for all sampling dates.

Random amplified polymorphic DNA profiles were analyzed by RAPDistance, v. 1.04 (Armstrong et al., 1996). The program RAPDSTAT was used to produce statistics about the populations for each quadrat, listing sample genotypes identical to other samples from the study. Euclidean distance matrices calculated for all populations were evaluated by AMOVA, v. 1.55 (Excoffier, 1995).

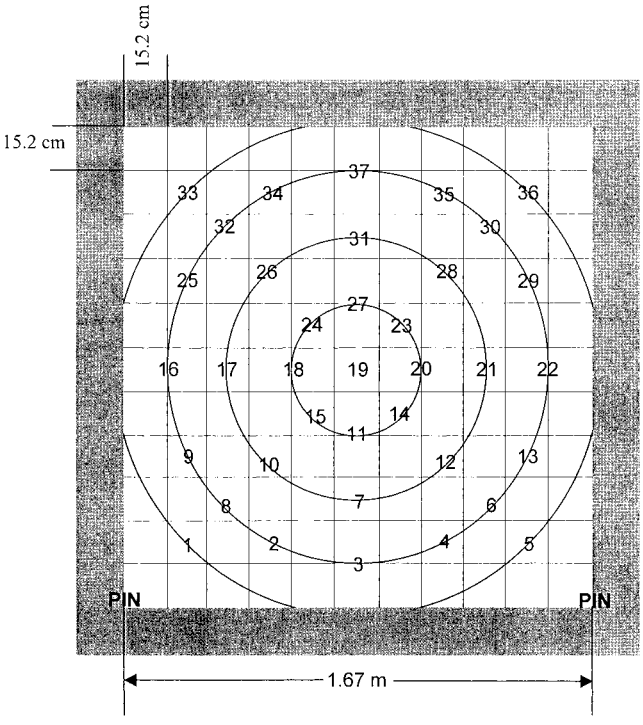

Fig. 1. The quadrat grid design for sampling white clover plants at the permanent quadrat locations. The numbers indicate the exact spot where leaves were collected if present. The sampling points were defined by the center of the quadrat (sampling point 19) and numbered sampling points on four concentric circles with radii of 22.8, 45.6, 68.4, and 85.6 cm, respectively. PIN, position of permanent steel pins in the field, was used for positioning the grid.

We used molecular markers to identify and track physical and temporal positions of clones and to characterize genetic variation of white clover populations at the local scale. Analyses of the RAPD profile data enabled us to estimate the number and size of clonal patches. However, with our sampling method, we could not determine the absolute size of clones, we could only estimate their size based on the number of samples with identical RAPD profiles. We assumed that samples with identical RAPD profiles were from the same ancestral plant and were thus a member of a clone. Analyses of molecular variance were calculated with Euclidean distance matrices based on RAPD profile data. This allowed us to infer effects of clonal structure and size on genetic variance within white clover populations. Analysis of molecular variance apportions the genetic variance among individuals within populations, among populations within groups, and among groups. Because AMOVA could not analyze more than 255 samples in an analysis, we only determined variances for the first two of the three levels of hierarchical analysis of molecular variance.

Repeated measures analysis of variance using SAS PROC MIXED (SAS Institute Inc., 1998) was used to analyze the data. The statistical model included years, sites, and sampling dates. Data were checked for normality and heteroscedasticity, resulting in assessment of significance on transformed scales for members per clone ($\log_{10}$) and percent of white clover (quartic root). Covariance structures for the repeated measures analyses were adequately modeled by either compound symmetry (samples per quadrat, clones per quadrat, genotypes per quadrat, single genets, and sward gap area) or heterogeneous compound symmetry (single genets, percent of white clover, and soil water). A compound symmetry structure has constant variance and covariance for all months, while a heterogeneous compound symmetry structure allows different variance and covariance for all pairs of months. Model filling criteria calculated by PROC MIXED were used to choose the best of several covariance structures. Tukey's method was used to adjust *P*-values for multiple means comparisons. Significance was assessed at the 5% level, unless indicated otherwise in the text.

RESULTS

In 1997, volumetric soil water content averaged for quadrats and dates on the JU pastures (0.19 $m^3\ m^{-3}$; Table 2) was lower ($P < 0.001$) than on the other two pastures (HU 0.25 $m^3\ m^{-3}$, MI 0.27 $m^3\ m^{-3}$; Table 2). When soil water values were averaged across three sites, there were differences among harvest dates ($P < 0.02$). There was no significant change from June to July 1997. In the 1998 growing season, the range of soil water content values was similar to that in 1997. The mean soil water values for the three sites in June, July, and September (0.13 to 0.19 $m^3\ m^{-3}$) were not different from each other, but were higher than in August (0.09 $m^3\ m^{-3}$), and lower than May (0.29 $m^3\ m^{-3}$) ($P < 0.001$). Soil water averaged across the three pastures was lower in 1998 (0.17 $m^3\ m^{-3}$) than in 1997 (0.24 $m^3\ m^{-3}$) ($P < 0.001$), even though precipitation amounts from January through August were 32% higher for the HU site, and 49% higher for MI and JU sites in 1998 than in 1997.

Table 2. Volumetric soil water content to a 15-cm depth on three Pennsylvania pastures by harvest date and year.

Site	May	June	July	Aug.	Sept.	
	$m^3\ m^{-3}$					Pooled SE§
			1997			
HU†	0.238‡	0.198	0.241	0.309	0.240	0.008
JU	0.196	0.183	0.089	0.254	0.214	
MI	0.327	0.259	0.282	0.237	0.265	
Pooled SE	0.005	0.010	0.007	0.005	0.008	
			1998			
HU	0.260	0.165	0.181	0.130	0.239	0.018
JU	0.316	0.113	0.142	0.070	0.098	
MI	0.309	0.100	0.176	0.062	0.219	
Pooled SE	0.026	0.013	0.013	0.015	0.014	

† HU, Huntingdon County; JU, Juniata County; MI, Mifflin County.
‡ Each value is the mean of 10 measurements of volumetric soil moisture content determined to a 15-cm depth and averaged for four locations on the pasture.
§ Applies to means within rows, within a site.

Most of the above normal precipitation in 1998 fell during January through April.

Floristic cover and sward gap area were variable within each quadrat at all sampling dates for both years (data not shown). The values for white clover cover were not normally distributed and were transformed to $(y+1)^{1/4}$ values. White clover cover for all sites was greater ($P = 0.0001$) in 1997 (21%) than in 1998 (10%). Sward gap area for all sites was lower ($P < 0.0001$) in 1997 (32%) than in 1998 (42%). Sward gap areas were highly variable in subquadrats and were as high as 95%. Although there were significant year effects for both variables and a significant site effect for white clover cover, no trends were apparent. No white clover seedlings were observed at any sampling date.

Of the 2133 samples taken, we found 1671 different RAPD profiles, or 1671 different genotypes. Of those genotypes, 1451 were sampled only once, and 220 were sampled more than once in the populations. Even though all identified genotypes were likely white clover clones, for the purposes of this report, the 1451 genotypes are called nonclonal samples and the 220 genotypes are called sampled clones. The number of sampled clones per quadrat varied from 0 to 6 (Table 3) when averaged across four quadrats per pasture. More sampled clones were identified (162 vs. 58) on the three pastures in 1997 than in 1998 ($P < 0.001$; see Table 3). The mean number of sampled clones per quadrat per year averaged across three pastures in 1997 and 1998 were 2.5 (SE 0.3) and 1.0 (SE 0.3), respectively. Furthermore, the number of sampled clones identified during a year differed among pastures ($P < 0.01$) and among dates of harvest ($P < 0.03$; Table 3). The fraction of clonal samples relative to the total number of samples ranged from 0.03 to 0.78 in 1997, and 0.04 to 0.33 in 1998.

The number of members sampled per clone on the three pastures for both years ranged from 0.5 to 4.2 in 1997, and from 0.5 to 2.2 in 1998 (Table 3). The values for this variable were not normally distributed, and were transformed to $\log_{10}$ values. Members sampled per clone varied among pastures ($P = 0.004$), within years ($P = 0.02$), and between years ($P < 0.0001$). The number of members detected for all sampled clones was 547 in 1997, and 135 in 1998. Most clones were detected at one date only, but nine and three clones were detected on two dates during 1997 and 1998, respectively. Members of eight and five clones were also found in different quadrats during 1997 and 1998, respectively.

Table 3. Number of clones† and members‡ per clone, averaged over four quadrats on three Pennsylvania pastures, based on RAPD profiles of leaf samples.

	Clones						Members per clone				
Site	May	June	July	Aug.	Sept.	SE§	May	June	July	Aug.	Sept.
					1997						
HU¶	0.2	3.8	0	1.0	2.2	0.4	0.5	4.1	0	1.0	1.6
JU	1.2	2.5	1.5	2.0	3.2		1.5	1.2	2.1	2.9	2.1
MI	2.8	4.2	3.5	6.0	3.2		3.7	3.1	2.4	4.2	12.8
SE#			0.5		0.6		††				
					1998						
HU	0	0.2	0	0.2	0	0.4	0	0.5	0	1.0	0
JU	1.5	1.8	0.5	2.5	0.2		1.6	1.8	1.0	2.2	0.5
MI	1.2	2.0	1.8	1.2	1.2		1.2	2.2	1.3	1.9	1.6
SE			0.5				††				

† Clone, a group of one or more genotypically identical samples.
‡ Members, samples with the same RAPD profile (genotype).
§ SE, pooled standard error. Applies to means within rows, within a site.
¶ HU, Huntingdon County; JU, Juniata County; MI, Mifflin County.
# Pooled standard error applies to means within columns, within a month.
†† Since significance was assessed on transformed data for members per clone, untransformed values are presented and SE values are not listed.

Comparison of RAPD profiles from 1997 samples with those from 1998 revealed only a few genotypes in both years. Presumably, most genotypes had survived the winter, but only a few were detected in both fall and spring sampling. Six clones found on a pasture in 1998 matched clones found on the same pasture in 1997. In each instance, members detected in both years were located in a different quadrat in the second year. Clones found in both years were never found in more than one quadrat during the second year. In 1997 and 1998, 28 and 16 members, respectively, were found in different quadrats. Two were found in two other quadrats.

The number of nonclonal samples on each site averaged across four quadrats ranged from 7 to 20 in 1997 and from 3 to 25 in 1998 (Table 4). Within each year, the HU pasture had fewer nonclonal samples (5 and 5) than the JU and MI ($P < 0.04$) pastures, which had 13

Table 4. Number of samples collected and number of nonclonal samples† averaged for four quadrats on three Pennsylvania pastures based on RAPD profiles of leaf samples.

	Samples						Nonclonal samples					
Site	May	June	July	Aug.	Sept.	SE‡	May	June	July	Aug.	Sept.	SE¶
						1997						
HU§	8	21	2	7	13	2.8	7	5	2	5	6	2.0
JU	19	26	14	18	18		16	20	10	12	8	
MI	23	32	27	31	32		12	18	18	8	7	
SE¶			2.1						1.8			
						1998						
HU	6	4	5	4	4	2.8	6	4	5	3	4	2.0
JU	24	19	21	16	10		20	16	20	11	10	
MI	28	24	26	22	29		25	20	22	18	25	
SE¶			2.1						1.8			

† Nonclonal samples, only one member detected.
‡ SE, pooled standard error. Applies to means within rows, within a site.
§ HU, Huntingdon County; JU, Juniata County; MI, Mifflin County.
¶ Pooled standard error applies to means within columns, within a month.

and 15 (JU) and 12 and 22 (MI). The number of samples per quadrat on each site ranged from 2 to 32 and 4 to 29 in 1997 and 1998, respectively (Table 4). There was no difference between years for the HU and JU sites, but the number of nonclonal samples was higher in 1998 than in 1997 for the MI site ($P = 0.0001$).

Genetic variances of individuals within and among populations on each of the three pastures was calculated with AMOVA for five harvest dates of each year (Table 5) and for four quadrat locations (Table 6) of each year. Not only did population variance change with time (Table 5), but population variance was variable among quadrats in a pasture (Table 6). Nearly all genetic variances were lower in 1997, and nearly all Φ_{st} values (correlation of random genotypes within populations relative to that of random pairs of genotypes drawn from the whole species) were higher in 1997 compared with 1998 (Tables 5 and 6). However, populations sampled in July and August from the HU pasture had higher genetic variances and lower Φ_{st} values in 1997 than in 1998 (Table 5).

On 8 of the 30 sampling events, two of the four quadrats on the pastures had populations with essentially the same genetic makeup (AMOVA data not shown). For example, in September 1998, quadrats 2 and 3 at the HU pastures shared a Φ_{st} of 0.03, which means the two populations were indistinguishable from each other. However, the probability that the variance within the populations (42%) and the Φ_{st} values were more extreme than randomly permuted values was low ($P = 0.10$). There were no instances where harvested samples from all four locations represented populations with similar genetic makeup. Furthermore, there were no instances where harvested samples from two different dates on the same or different pastures revealed populations with similar genetic makeup. Analysis of molecular variance results indicated that at the local scale white clover genetic variance in quadrat populations (Table 6) did not drop to low levels (less than 10%) across two successive growing seasons.

Table 5. Analysis of molecular variance (AMOVA) of white clover populations in four quadrats by harvest date in three Pennsylvania pastures based on RAPD profiles of leaf samples.

	1997			1998		
Month	Within populations†	Φ_{st}‡	n	Within populations	Φ_{st}	n
	%		HU§	%		
May	54	0.46	31	80	0.20	25
June	40	0.60	39	76	0.24	18
July	64	0.36	7	54	0.46	21
Aug.	74	0.26	28	46	0.54	16
Sept.	42	0.58	47	70	0.30	16
			JU			
May	59	0.41	75	76	0.24	95
June	64	0.36	103	67	0.24	76
July	54	0.46	54	78	0.22	83
Aug.	51	0.49	71	70	0.30	66
Sept.	58	0.42	73	71	0.28	41
			MI			
May	36	0.64	92	67	0.33	113
June	44	0.56	126	52	0.48	96
July	40	0.60	108	63	0.37	102
Aug.	45	0.55	125	60	0.40	86
Sept.	15	0.85	130	70	0.30	117

† Variance of individuals among populations is 100 minus the variance of individuals within populations.
‡ Φ_{st}, the genetic variance calculated by analysis of molecular variance (AMOVA), is the correlation of random genotypes within populations relative to that of random pairs of genotypes drawn from the whole species.
§ HU, Huntingdon County; JU, Juniata County; MI, Mifflin County.

Table 6. Analysis of molecular variance (AMOVA) of white clover populations in five dates by quadrat in three Pennsylvania pastures based on RAPD profiles of leaf samples.

	1997			1998		
Quadrat	Within populations†	Φ_{st}‡	n	Within populations	Φ_{st}	n
	%		HU§	%		
1	25	0.75	57	–	–	–
2	31	0.69	132	63	0.37	25
3	45	0.55	158	52	0.48	25
4	37	0.63	41	56	0.44	14
			JU			
1	43	0.57	109	52	0.48	58
2	51	0.49	219	62	0.38	129
3	48	0.52	191	66	0.34	122
4	56	0.44	144	62	0.38	52
			MI			
1	34	0.66	240	62	0.38	158
2	36	0.64	252	61	0.39	126
3	33	0.67	238	60	0.40	121
4	46	0.54	201	57	0.43	110

† Variance among populations is 100 minus the variance within populations.
‡ Φ_{st}, the genetic variance calculated by analysis of molecular variance (AMOVA), is the correlation of random genotypes within populations relative to that of random pairs of genotypes drawn from the whole species.
§ HU, Huntingdon County; JU, Juniata County; MI, Mifflin County.

DISCUSSION

Change in genotypic composition of a white clover population with time is a function of seedling recruitment, vegetative growth of plants, death of rooted stolons, and death of clones. The use of RAPD profiles enabled us to follow physical and temporal positions of some genotypes and to characterize genetic variation of white clover populations at the local scale. The data also allowed us to estimate the number and size of clonal patches. We found that the size and number of clones and the number of nonclonal samples were essentially unchanged across 2 yr (Tables 3 and 4). Our results extend previous reports on white clover genetic diversity in pastures. Earlier work established higher than expected genetic variability of this species on a regional scale (Gustine and Huff, 1999), a permanent grassland scale (Burdon, 1980), and a local scale (Gustine and Sanderson, 2001), a seeming contradiction of its stoloniferous growth habit.

The use of RAPD profiles based on stable DNA markers allowed us to identify white clover plants belonging to the same clone, because those sampled trifoliate leaves had identical genotypes. Approaches previously taken to understand how white clover populations maintain high levels of genetic diversity include: (i) locate and measure obvious clones in a grassland using genetically controlled morphological traits such as leaf-

mark patterns (Brewbaker, 1955; Carnahan et al., 1955); (ii) cross pollinate suspected clonal individuals that would be self-sterile (Harberd, 1963); or (iii) identify and track genotypes throughout the growing season at specific points on a grid placed in a field (Cahn and Harper, 1976). We found that some sampled clones were detected more than once in the same or a different quadrat. A genotype could have been missing because trifoliate leaves on the same stolon had been grazed at the sampling point, a different stolon was sampled, or the stolon was dormant. However, if clones were sampled later in the same quadrat, members were often distributed differently within the quadrat, and the number of members was changed (Gustine and Sanderson, 2001). This observation may suggest that some clones were dormant during part of the growing season. White clover stolons that became dormant under severe drought resumed growth when soil water was restored (Harberd, 1963).

Analysis of molecular variance (Table 5) showed that at the farm scale, within-population genetic variability of white clover populations containing clones ranged from 15% (highly clonal) to 80% (few clones). The within-population genetic variances were generally lower in 1997 than the 60 to 75% reported by Gustine and Huff (1999), but were generally within that range in 1998. This indicated that differing management practices on the 3 farms were less important in affecting population genetic variance than differences in weather. The genetic variances within populations by quadrat (Table 6) and by harvest date (Table 5) were 15 to 80%, suggesting that genotypic heterozygosity was maintained even though most populations had one or more clones present. We also found that no two of the three white clover populations in Pennsylvania pastures had similar genetic makeups when sampled on the same date (Table 5). In previous research, Gustine and Huff (1999) demonstrated that the genotypic composition of four Pennsylvania populations changed during a 6-wk period (Gustine and Huff, 1999) and that the genetic composition of three Pennsylvania populations changed frequently throughout the 1997 growing season (Gustine and Sanderson, 2001). Tables 5 and 6 demonstrate that the genetics of these white clover populations were also variable with time and space in quadrats on differently managed farms. Variations in the environment, changes in soil fertility, and appearance of new sward gap areas because of grazing may be the driving force for this.

We did not observe seedling white clover plants in the subquadrats during the study, yet we documented variability in the genetic makeup of the populations within quadrats during two growing seasons. Seedling recruitment, however, can not be ruled out. The combination of new genotypes and intermittent growth of stolons (Harberd, 1963) could account for the changing genetic composition of the white clover populations detected by our sampling method.

A body of evidence indicates that although seedling recruitment rates are low in clonal plant species (Barratt and Silander, 1992), high levels of intraspecific genetic diversity are often found (Ellstrand and Roose, 1987; McClellan et al., 1997; Widen et al., 1994). This situation was also reported for white clover (Burdon, 1980; Turkington, 1985). Theories to explain this apparent paradox rely on environmentally driven changes in expressed morphology of the plants. This could account for phenotypic variation, but not for genotypic variation. Somatic mutations (Turkington, 1985; Silvertown and Lovett Doust, 1993) in white clover could add to genetic variability detected with RAPD profiles. Additional research with molecular markers to identify genotypes of leaf and stolon tissues of individual clones is necessary to demonstrate somatic differences within a white clover plant.

Because AMOVA within-population genetic variances ranged from 15 to 74% in 1997, and 46 to 80% in 1998, we conclude that genetic diversity persists in populations with a high incidence of clones. We also found that the fraction of clonal samples sampled relative to the total number of samples was higher in 1997 than in 1998. We therefore suggest that higher numbers of clonal members and lower within-population variances in 1997 were consistent with reduced genetic diversity in highly clonal populations.

CONCLUSIONS

Several mechanisms of white clover vegetative reproduction may have been at work to prevent low genetic variation in the populations. Our results suggest that changes in area covered and position of clonal patches contribute to variation in white clover populations. Although we did not demonstrate that temporal dormancy of white clover plants is a factor, this may also contribute to the dynamic genotypic diversity of white clover, along with loss of plants by winter kill and rare seedling recruitment.

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